Comparison of the Offspring Sex Ratio Between Cleavage Stage Embryo Transfer and Blastocyst Transfer

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SUMMARY

Objective: To compare the sex ratio of offspring born after cleavage stage embryo transfer and blastocyst transfer.
Materials and Methods: In this retrospective study of embryo transfer (ET), we included 473 offspring from 446 deliveries during the period January 2002 to December 2007. Statistical analysis was performed on the sex ratio of offspring resulting from day 3 cleavage stage embryo transfer and from sequential blastocyst culture transfer.
Results: In total, 446 patient deliveries were included in this analysis. There were 251 singleton pregnancies, 109 twin pregnancies, and four triplet pregnancies. The total number of offspring was 473, of which 118 resulted from day 3 ETs, and 355 resulted from blastocyst ETs. At our center, the influence on the sex ratio of cleavage stage ET and blastocyst-stage ET showed a bias towards males in both cases. The overall female to male ratio for offspring resulting from day 3 ETs was not significantly higher than the same ratio for offspring resulting from blastocyst ETs ($p = 0.24$; odds ratio, 0.762). The female to male ratio for either singleton births or multiple deliveries was also not significantly different between day 3 ETs and blastocyst ETs.
Conclusion: The sex ratio was influenced by cleavage stage ET and blastocyst-stage ET. In both cases, there was a bias towards males. In addition, when blastocyst ET was compared with day 3 ET, there was no further increase in the percentage of male offspring. [*Taiwan J Obstet Gynecol* 2010;49(1):35–39]

Key Words: blastocyst transfer, cleavage-stage embryo, embryo morphology, sex ratio

Introduction

According to an analysis of the results from assisted reproductive techniques (ART) in Taiwan from the Bureau of Health Promotion, Department of Health, from 2002 to 2006, the overall sex ratio of offspring born using ART favored males (53.2% *vs.* 46.8%). During this period, the annual sex ratio of offspring from ART remained almost constant, and all the ratios were observed to be biased towards males.

The sexual imbalance related to *in vitro* fertilization (IVF) and embryo transfer (ET) has been discussed previously in the literature. As is well known, the human sex ratio at birth has been found to be related to a variety of preconception and intrapartum factors. Previous animal studies have shown that the factors may range from genetics to the differential survival of male fetuses *in utero* [1–5].

During IVF, the current selection criteria for human embryos that are to undergo transfer is often based on morphologic criteria and the development rate. Some reports have suggested that male embryos develop faster in culture than female embryos and that the use of blastocyst culture for embryo selection will result in a sex

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ratio imbalance in favor of male offspring [6,7]. Other studies have shown the opposite result and reported no developmental difference between male and female embryos [8–10]. The latest meta-analysis study [11] of impact of blastocyst transfer (BT) on offspring sex ratio has shown that BT appears to be associated with a sex ratio skewed more in favor of males than cleavage stage ET, and that there is also an increased chance of monozygotic twins. In addition, preimplantation genetic diagnosis has also been applied to examine the sex ratio during IVF [9]. This study concluded that blastocyst stage ET does not influence the live birth sex ratio of embryos when these are assessed using normal preimplantation fluorescent *in situ* hybridization genetic screening.

The objective of this analysis was to evaluate the effect of cleavage embryo and BT during IVF on the sex ratio of offspring at our center. Furthermore, we also examined the relationship between embryonic grading of the cleavage embryos and blastocysts and its effect on the sex ratio of offspring.

Materials and Methods

Patients

Data were collected from consecutive infertile couples who underwent ART and transcervical ET between January 2002 and December 2007. No patients underwent fluorescent *in situ* hybridization genetic preimplantation genetics screening for sex selection. Intracytoplasmic sperm injection cycles were also excluded. We retrospectively analyzed all offspring born because of embryo transfer performed at our center. During the study period, 446 patient deliveries were included in this analysis. These 251 singleton pregnancies, 109 twin pregnancies, and four triplet pregnancies were analyzed. The overall number of offspring was 473, of whom 118 resulted from day 3 ETs, and 355 resulted from blastocyst ETs. The institutional review board of the ethics committee of Chang Gung Memorial Hospital approved this study.

Controlled ovarian hyperstimulation and oocyte retrieval

The protocol for controlled ovarian hyperstimulation followed the standard downregulation regimen, as we have previously reported [12,13]. Briefly, all women received either the long or short protocol of pituitary downregulation with leuprolide acetate (Lupron; Takeda, Tokyo, Japan) depending on ovarian reserve, as assessed by the patient's age, baseline serum follicle-stimulating hormone (FSH) concentration, or previous response to ovarian stimulation. Exogenous FSH was administered at an initial dose of 150–300 IU, with further doses given according to the individual's ovarian response, as assessed by serum estradiol level determination and sonographic follicular growth monitoring. When the lead follicle reached 16–18 mm in diameter, leuprolide acetate and FSH were discontinued, and human chorionic gonadotropin (hCG) was administered. Oocyte retrieval was performed by transvaginal ultrasound-guided follicle aspiration 36–38 hours after hCG administration. All of the retrieval procedures were performed by clinical reproductive endocrinologists. A single team of embryologists coordinated all procedures, thereby ensuring that both the culture protocols and the embryo assessment were standardized.

Semen evaluation and preparation

Sperm concentration and motility were evaluated according to World Health Organization recommendations. Husbands were asked for fresh ejaculate on the day of oocyte retrieval. The ejaculate specimens were prepared using the Percoll (Sigma, St. Louis, MO, USA) gradient method as we have previously described [14].

Oocyte preparation, assessment of fertilization, and embryo culture

Standard IVF procedures were used to achieve oocyte fertilization. Gametes were fertilized in universal IVF medium (Medi-Cult, Jyllinge, Denmark), and fertilization was evaluated 16–18 hours after IVF. Normal fertilization was defined as zygotes with two pronuclei (2PN) after IVF. Fertilization failure was defined as zero oocytes achieving zygote stage with 2PN. Normal fertilization rate was reported as a percentage of total number of oocytes undergoing IVF. The zygotes with 2PN were cultured for another 48 hours in 100 μL microdrops of G1 medium (Vitrolife, Göteborg, Sweden) under oil. Zygotes with 2PN were cultured until the day of ET. G1TM medium (Scandinavian IVF Science, Göteborg, Sweden) was used for culture of embryos on day 1 to day 3. G2TM medium (Scandinavian IVF Science) was used for culture of embryos from day 3 to day 5 or day 6. In our program, we have routinely offered BT to patients with more than three eight-cell embryos on day 3. The zygotes were scored according to the *Z* score scoring system [15]. Zygotes with equal numbers of nucleolar precursor bodies aligned on the pronuclear junction were designated Z-1. Those with equal numbers of nucleolar precursor bodies that were scattered were designated Z-2. Those with inequality of numbers or alignment were designated Z-3, and those with very unequally sized pronuclei or pronuclei that were not aligned in a central position within the oocyte were designated Z-4. Veeck's morphologic

grading system was adopted for day 3 embryo scoring [16]. Pre-embryos with eight cells, blastomeres of equal size and no cytoplasmic fragments were scored as grade I. Pre-embryos with eight cells, blastomeres of equal size and minor cytoplasmic fragments were scored as grade II, and those with blastomeres of distinctly unequal size and no cytoplasmic fragments were scored as grade III. Pre-embryos with four to eight cells and moderate to heavy fragmentation were scored as grade IV. Pre-embryos with few blastomeres of any size and with major and complete fragmentation were scored grade V. We defined "good embryos" as zygotes that had *Z* score grade 1 and Veeck's scoring system on day 3 embryo grade 1. All ETs were performed gently using a Labotect catheter (Labotect, Göttingen, Germany).

Luteal phase support

Luteal phase supplementation of micronized progesterone was started on the day of oocyte retrieval and continued until the day pregnancy was confirmed by detecting hCG in the urine. The study population received Crinone 8% gel (Fleet Laboratories Ltd., Watford, UK; 90 mg daily) or received Utrogestan vaginal capsules (Piette International Laboratories, Belgium; 200 mg four times daily). These two formulas were obtained from the hospital during the study period. However, the choice of a particular formula depended on the affordability to the patient and convenience of application. The day following BT, we offered a single dose, 250 μg recombinant hCG booster (Ovidrel; Industria Farmaceutica Serono SpA, Italy) [17].

Statistical analysis

The SigmaStat statistical package (Jandel Corp., San Rafael, CA, USA) was used for data analysis. Continuous data were summarized as mean ± standard deviation. The study included the Mann-Whitney rank sum test for comparison of means and Fisher's exact test for proportions. All *p* values were two-sided, and a *p* value of < 0.05 was considered statistically significant.

Results

General characteristics

In total, 446 patient deliveries were included in this analysis. There were 251 singleton pregnancies, 109 twin pregnancies, and four triplet pregnancies. The overall number of offspring was 473, of whom 118 resulted from day 3 ETs, and 355 resulted from BTs. No significant differences were noted between the cleavage ET and BT with the regard to patient age, body mass index, endometrial thickness on day of hCG, estradiol on hCG day, fertilization rate, and number of embryos transferred (Table 1). The only significant value was the greater numbers of oocytes retrieved in the BT group ($p < 0.001$). Fetal reduction was performed in two cases among the cleavage stage ET group (3–1 = 2) and 16 cases among the BT group (13 cases, 3–1 = 2; two cases, 4–2 = 2; one case, 2–1 = 1). The fetal reduction rates were low in these two groups (1.6% [2/128] *vs.* 5.1% [16/315]).

Female to male ratios of cleavage stage ET and BT groups

When the sex ratio was analyzed according to the number of offspring born per delivery, we found a sex ratio imbalance for overall deliveries towards males of 60.17% when day 3 ETs were performed, compared with 53.6% when BTs were used. However, the female to male (F:M) ratios were not significant between the two groups (1:1.516 for day 3 ETs, compared with 1:1.15 for BTs [$p = 0.24$; odds ratio, 0.762; 95% confidence interval, 0.499–1.164; Table 2]). When the subgroups involving singleton or multiple deliveries were compared, the F:M ratio was also not significant between the two groups.

Table 1. Laboratory and clinical data for the transfer cycles*

	Cleavage stage embryo transfer	Blastocyst transfer
No. of cycles	128	315
Age of female partners (yr)	33.5 ± 4.1	32.2 ± 4.1
Body mass index (kg/m^2)	22.1 ± 3.3	21.6 ± 3.4
Endometrial thickness on day of hCG (mm)	13.1 ± 0.2	13.3 ± 0.2
Estradiol (pg/mL) on hCG day	1,204 ± 927.8	1,957 ± 1,033.5
Normal fertilization rate (%)	84.83	89.55
No. of mean embryos transferred	2.67 ± 0.82	2.64 ± 0.64
No. of oocytes retrieved	4.1 ± 2.3†	8.6 ± 3.4†
Implantation rate, *n* (%)	158/346 (45.6%)	431/838 (51.4%)

**Data are presented as n or mean ± standard deviation; †p < 0.001. hCG = human chorionic gonadotropin.*

Good embryos transferred during cleavage stage ET and BT

The percentage of good embryos among the cleavage stage ET groups was 53.7% (187/348; mean numbers, 1.46 ± 0.93), while the percentage of good embryos among the BT group was 79.1% (663/838; mean numbers, 2.10 ± 0.67). Thus, the mean numbers of good embryos was significantly higher among the BT group, as expected ($p < 0.001$; Table 3).

Discussion

The sex ratio for all births reported in Taiwan in 2008 favored males at 109.64. During the period from 2002 to 2006, the overall sex ratio of offspring born using ART in Taiwan also favored males (53.2% *vs*. 46.8%). The data from our study over the period 2002 to 2007, which compared the sex ratio for cleavage ET and BT births that took place as part of the same program and over the same period, showed a trend in favor of male offspring. The sex ratio of infants born as a result of BT favored males at 115.1, but this was not significantly different from the sex ratio for early cleavage stage ET, which also favored males at 151.0. Furthermore, a similar trend was also observed for both the singleton and multiple pregnancy subgroups. Thus, it would appear that the sex ratio after both cleavage stage embryo and BT is biased towards males. In addition, our data did not show a significantly increased sex ratio in favor of males with BT compared with cleavage stage ET, which is contrary to results reported by Chang et al [11] in the latest meta-analysis. Transfer details, including the different selection criteria used in the different studies, and the number of transfers are not clear in the meta-analysis. We think that these factors must be taken into consideration and the number transferred may also need to be corrected.

Prior data using murine, bovine and porcine models [1,2] have suggested that male embryos develop at a faster rate than female embryos and, therefore, that the fastest cleaving embryos tend to be male. In humans, male live births were six times higher when the mean number of cells was more than four at the time of ET on day 2 [18]. Similarly, Ray et al [6] showed that male embryos cultured to the blastocyst stage had a greater number of cells on day 2 than female embryos and that this difference was maintained up to the blastocyst stage in both the trophectoderm and inner cell mass. Previous studies have reported some embryo selection parameters related to the sex ratio difference, such as embryo grading (expansion, trophectoderm and inner cell mass) [7] and the daily rate of embryonic development [8,10]. However, there is presently no conclusive morphologic parameter that is able to predict the sex of an embryo.

Previous studies have shown that *Z* score and the morphologic grade of the embryos on post-conception day 3 are highly predictive of the implantation rate and live birth rate [15,19]. Therefore, it is reasonable that the sex ratio of offspring should be highly correlated with good embryo transfer. At our center, preference for

Table 2. Sex ratio according to cleavage embryo transfer and blastocyst embryo transfer in singleton and multiple pregnancies*

	Cleavage stage embryo transfer		Blastocyst transfer		*p*	OR	95% CI
	Female	Male	Female	Male			
All deliveries ($n = 473$)	47 (39.8)	71 (60.2)	165 (46.5)	190 (53.5)	0.240	0.762	0.499–1.164
Singleton ($n = 251$)	28 (37.8)	46 (62.2)	80 (45.2)	97 (54.8)	0.328	0.738	0.424–1.286
Multiple ($n = 222$)	19 (43.2)	25 (56.8)	85 (47.8)	93 (52.2)	0.616	0.832	0.428–1.617

**Data are presented as n (%). OR = odds ratio; CI = confidence interval.*

Table 3. Percentage of good embryos transferred and number of mean top-quality embryos transferred

	Cleavage stage embryo transfer	Blastocyst transfer
Good embryos transferred, *n* (%)	187/348 (53.7*)	663/838 (79.1*)
Good embryos transferred, mean ± SD	1.46 ± 0.93*	2.10 ± 0.67*

**p < 0.001. SD = standard deviation.*

transfer is given to good embryos selected as showing Z-1 and eight cells, and grade 1 according to day 3 embryo morphology. In this study, the percentage of such good embryos for cleavage stage embryos transfer was 53.7%, but was 79.1% in BT group.

A higher percentage of good embryos for either cleavage stage ET or BT may result in a higher implantation rate. Therefore, the sex ratio bias towards males and good embryo selection may be correlated. According to our results, the current selection policy, which favors good embryos for transfer at our center, may overlap with the morphologic characteristics that predict sex. Therefore, the sex ratio could be biased towards males whether cleavage stage embryos or BT was chosen.

The first limitation of this study is that we always transferred more than one embryo, so the sex of the live-born infant cannot be correlated precisely with embryonic development in the laboratory. Another limitation is that we do not know if female/male embryos have different survival rates in the stimulated environment after controlled ovarian hyperstimulation, and this may have affected our results. Further studies are needed to confirm the relationship between sex and embryo quality using preimplantation genetic diagnosis. In addition, the overlap between our selection criteria and the morphologic characteristics that predict sex also needs additional study. Finally, studies involving a larger number of patients are needed to verify our results.

In conclusion, this study has demonstrated that it is not cleavage ET or BT themselves that alters the sex ratio, but perhaps the selection criteria used during ET, which favor good embryos for transfer. This would seem to lead to a significant shift in the sex ratio for both cleavage stage ET and BT.

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